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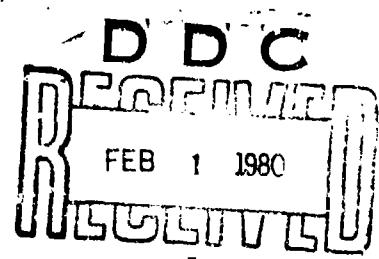
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MAMMALIAN TOXICITY OF MUNITIONS COMPOUNDS

Summary of Toxicity of Nitrotoluenes

PROGRESS REPORT NO. 11

Contract No. DAMD-17-74-C-4073  
MRI Project No. 3900-B



For

Contract Officer's Technical Representative: Dr. Jack C. Dacre  
Environmental Protection Research Division  
U.S. Army Medical Bioengineering Research and  
Development Laboratory  
Fort Detrick, Frederick, Maryland 21701

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PROGRESS REPORT NO. 11

January 1980

by

Harry V. Ellis, III  
Chuen-Bin Hong  
Cheng-Chun Lee

Supported by

U.S. Army Medical Research and Development Command  
Fort Detrick, Frederick, Maryland 21701

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2,6-Dinitrotoluene (2-Methyl-1,3-dinitrobenzene, CAS Reg. No. 606-20-2)  
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3,5-Dinitrotoluene (1-Methyl-3,5-dinitrobenzene, CAS Reg. No. 618-85-9)  
2-Amino-4,6-dinitrotoluene (2-Methyl-3,5-dinitrobenzenamine, CAS Reg. No. 35572-78-2)  
4-Amino-2,6-dinitrotoluene (4-Methyl-3,5-dinitrobenzenamine, CAS Reg. No. 19406-51-0)

Toxicity

Irritation

Allergenicity

Carcinogenicity

Mutagenicity

Reproductive toxicity

Disposition

Metabolism

Excretion

Risk Assessment Water Quality Criterion

PREFACE

This report was prepared at Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110, under U.S. Army Contract No. 17-74-C-4073, MRI Project No. 3900-B, "Munitions Compounds Mammalian Toxicity Study." The work was supported by the Medical Research and Development Command, Department of the Army. Captain John P. Glennon and Dr. Jack C. Dacre of the Environmental Protection Research Division, USAMBRDL, were the successive Contract Officer's Technical Representatives for the project.

On February 20 to 22, 1979, we presented the findings of this project at a technology transfer meeting at USAMBRDL. At the request of the COTR, we have taken our presentation on nitrotoluenes and converted it to written form, as this report. This includes all significant findings on the mammalian toxicity of nitrotoluenes, including comparisons of the various compounds and acceptable extrapolations, with references to the Progress Report giving the full details and negative results.

Approved for:

MIDWEST RESEARCH INSTITUTE

*Harry L. Ellis*  
for Cheng-Chun Lee  
Principal Advisor for  
Pharmacology/Toxicology

January 18, 1980

## ABSTRACT

This report is a summary of all findings on nitrotoluene derivatives done in the course of the contract. It incorporates portions of Progress Reports Nos. 1, 3, 4, 6 and 7, as well as overall comparisons and conclusions.

Acute toxicity data, including rodent LD<sub>50</sub>s, rabbit irritation tests, guinea pig dermal sensitization test, single-dose metabolism study in rats, and the Ames Salmonella/microsome test were done on 2,4,6-trinitrotoluene, all dinitrotoluene (DNT) isomers (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-) and (except for the metabolism study and Ames test) 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Subchronic toxicity tests were done with 2,4-DNT and 2,6-DNT in dogs, rats and mice. Chronic toxicity tests were done with 2,4-DNT in all three species, accompanied by reproductive studies in rats. The metabolism of 2,4-DNT was studied in rats after chronic feeding of 2,4-DNT and in mice, rabbits, dogs, monkeys and in vitro preparations in single-dose studies.

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## I. INTRODUCTION

Under Contract No. DAMD-17-74-C-4073, entitled "Munitions Compounds Mammalian Toxicity Study," we have performed a variety of studies, divided into three phases. Phase I, Effects of Acute Exposure, includes acute oral toxicity, primary skin and eye irritation, dermal sensitization, and disposition and metabolism studies. Results on 2,4,6-trinitrotoluene and on some dinitrotoluene isomers were reported in Progress Report No. 1.<sup>1/</sup> Results on additional compounds plus in vitro mutagenic (Ames test) studies were reported in Report No. 6.<sup>2/</sup> Phase II, Effects of Multiple Exposure, includes subacute and subchronic toxicity, reversibility, immunologic response, chemical-biological interaction, mutagenicity, and disposition and metabolism studies. Results were presented in a series of reports. The compounds tested were trinitroglycerin (TNG),<sup>3/</sup> 2,4-dinitrotoluene (2,4-DNT),<sup>4/</sup> 2,6-dinitrotoluene,<sup>5/</sup> and nitrocellulose (NC).<sup>6/</sup> Phase III, Effects of Life-Time Exposure, includes chronic toxicity, reversibility, reproductive, cytogenetic, and metabolism studies on three of the compounds: 2,4-DNT,<sup>7/</sup> TNG,<sup>8/</sup> and NC.<sup>9/</sup> This report is a general summary and correlation of our results on nitrotoluenes. Methods and negative findings are only sketched here. Details are available in the original reports.<sup>1,2,4,5,7/</sup>

Nitrotoluenes are derived from the nitration of toluene. The classical process involves three separate reactions with progressively stronger mixtures of nitric and sulfuric acids to produce trinitrotoluene (TNT). A more recent process is continuous, using a single reactor. Normally, the first nitration will give about 96% of the ortho and para isomers of mononitrotoluene. Therefore, the second step produces 2,4-dinitrotoluene (2,4-DNT), the major product, 2,6-DNT, and a small amount of other isomers. The final step produces about 95% 2,4,6-trinitrotoluene ( $\alpha$ -TNT); the other isomers and products are removed in the purification of the TNT and are of no further concern to this study.

TNT is a major military explosive, especially as the main charge in shells, bombs and mines. It is being replaced, at least in part, by more powerful explosives (such as rdx0 and mixtures containing RDX and/or other explosives (cyclotol, composition C-4, etc.). 2,4-DNT has some use as an ingredient in propellant mixtures. 2,4-DNT and other DNTs, including mixtures, are produced commercially in large quantities. They are reduced to diaminotoluenes, reacted with phosgene to make toluediisocyanate and then polymerized to create polyurethane foams.

## II. MATERIALS AND METHODS

For details, consult the previous reports in this series.<sup>1,2,4,5,7/</sup>

### A. Materials

#### 1. Chemicals

The following compounds were studied. They were relatively pure, although some contained up to 2% impurities, generally identified as another isomer.

2,4,6-TNT.

DNTs: 2,3-; 2,4-; 2,5-; 2,6-; 3,4-; 3,5-.

Singly reduced isomers of 2,4,6-TNT: 2-ADNT (2-amino-4,6-dinitrotoluene); 4-ADNT (4-amino-2,6-dinitrotoluene).

#### 2. Animals

Strains used included CD<sup>®</sup> albino rats and CD-1<sup>®</sup> albino mice (Charles River Breeding Laboratories, Wilmington, Massachusetts), guinea pigs and New Zealand rabbits (Small Stock Industries, Pea Ridge, Arkansas), beagle dogs (Hazleton Research Animals, Cumberland, Virginia) and rhesus monkeys (Primate Imports, Port Washington, New York). Swiss mice, used in the acute toxicity study, and B6C3F1 mice, used in the metabolism study, were bought from Charles River Breeding Laboratories.

Dogs for the subacute and chronic studies were kept in indoor/outdoor runs. All other animals were kept caged in air conditioned rooms. The usual methods for laboratory animal care were followed.

### B. Methods

#### 1. Acute toxicity studies<sup>1,2/</sup>

Male and female rats and mice were given oral doses of the compound in peanut oil and observed for 14 days. LD<sub>50</sub>s were calculated by the probit method of Finney.

Primary skin and eye irritation were studied in rabbits by the Draize procedure.

Dermal sensitization was studied in guinea pigs by the maximal sensitization method.

2. Repeated dose studies<sup>4,5,7</sup>

a. Species and doses

The following species and doses were used.

Subacute study of 2,4-DNT

Dogs: 0, 1, 5 or 25 mg/kg/day (in capsule).

Rats and mice: 0, 0.07, 0.2 or 0.7% (in feed).

Subacute study of 2,6-DNT

Dogs: 0, 4, 20 or 100 mg/kg/day (in capsule).

Rats and mice: 0, 0.01, 0.05 or 0.25% (in feed).

Chronic study of 2,4-DNT

Dogs: 0, 0.2, 1.5 or 10 mg/kg/day (in capsule).

Rats: 0, 0.0015, 0.01 or 0.07% (in feed).

Mice: 0, 0.01, 0.07 or 0.5% (in feed).

b. Design

The following designs were used.

Subacute study

Group I: 4-week dosing.

Group II: 4-week dosing plus 4-week recovery.

Group III: 13-week dosing.

Group IV: 13-week dosing plus 4-week recovery.

Chronic study

Group I: 1-year dosing.

Group II: 1-year dosing plus 1-month recovery.

Group III: 2-year dosing.

Group IV: 2-year dosing plus 1-month recovery.

Each group at each dose included one male and one female dog or four male and four female rats or mice, except that there were two male and two female dogs per group for the 2 year and 25 month groups, and 30 male and 30 female rats, and 50 male and 50 female mice for the 24 month group.

c. Parameters

All dogs were periodically bled from their jugular vein for a hematology battery (including methemoglobin and Heinz bodies) and a clinical chemistry battery. Groups of four rats of either sex were periodically bled from their tails for hematology and from their aorta at termination for clinical chemistry. Groups of four mice of either sex were bled from their aorta at termination for hematology.

At termination, major organs were weighed; those organs, other organs and tissues, and any apparent lesions were prepared and examined microscopically. Moribund animals were killed and examined as if at termination.

Other parameters examined included behavior, body weight, feed consumption and the ancillary studies discussed below.

### 3. Mutagenesis studies

#### a. Ames test<sup>2/</sup>

The Salmonella/microsome plate test ("Ames test") was done by standard methods.

#### b. Cell cultures<sup>4,7/</sup>

Mutagenic effects were tested in Chinese hamster ovary cell cultures<sup>4/</sup> and in cultures of kidneys and lymphocytes<sup>4/</sup> or marrow cells<sup>7/</sup> of dogs and rats in the multiple dose toxicity studies.

#### c. Dominant lethal mutation studies<sup>7/</sup>

Four dominant lethal mutation studies using male rats fed various doses of 2,4-DNT and untreated female rats were done, following standard methodology.

### 4. Reproduction study<sup>7/</sup>

A three generation reproduction study using rats fed the same doses of 2,4-DNT as with the chronic study was done. Each successive generation was produced by the second litter of the previous generation.

### 5. Metabolism studies

#### a. In vivo studies

Rats<sup>1,2/</sup> were given single doses of the various ring-UL-<sup>14</sup>C compounds. Excretions were collected for 24 hr. Then the animals were killed and the levels of <sup>14</sup>C in the tissues and excreta determined. Parallel studies were done with 2,4-DNT in mice, rabbits, dogs and monkeys and the metabolites identified.<sup>4/</sup>

Single oral doses of the various radiolabeled compounds were given to rats, and the biliary excretion was determined.<sup>4/</sup>

Single doses of  $^{14}\text{C}$ -2,4-DNT were given to rats fed 2,4-DNT for 3, 9 or 20 months; the excretion and metabolism were monitored.<sup>7/</sup>

b. In vitro studies<sup>4/</sup>

The metabolism of 2,4-DNT by in vitro preparations of liver from rats, mice, rabbits, dogs and monkeys was studied.

c. Interactions<sup>4/</sup>

Zoxazolamine paralysis time and hepatic nitroanisole O-demethylase activity were used to determine the effect of 2,4-DNT on drug metabolizing enzymes.

### III. RESULTS AND DISCUSSION

#### A. Acute Toxicity Studies<sup>1,2/</sup>

##### 1. Rodent LD<sub>50</sub>s

The acute oral LD<sub>50</sub>s in rats and mice are summarized in Tables 1 and 2 and depicted graphically in Figures 1 and 2, respectively. The various compounds have similar potency, with TNT (1 g/kg) being about the middle of the range. 3,5-DNT is the most toxic, especially in rats; while 2-ADNT and 4-ADNT were the least toxic. There are some sex and species differences; mice are usually slightly less sensitive.

In most cases, the only toxic signs were central nervous system depression which, within a few hours, either produced ataxia, respiratory depression and death or ceased leading to complete recovery. TNT and 3,4-DNT caused some brief convulsions. The ADNTs caused delayed deaths, as late as 10 days after dosing. Rats were hyperexcitable after ADNT dosing; they seemed about to convulse, but never did. If stimulated, they showed extremely exaggerated reflexes. Red urine was produced by rats and mice given TNT; orange to yellow urines came from animals given 2,5-DNT, 3,4-DNT, 2-ADNT or 4-ADNT. In most cases, the urine was a more reddish color than the original compound, indicating metabolism of the given compound; in the other cases, the tints were identical.

##### 2. Irritation

Results of the primary skin irritation studies in rabbits are in Table 3. 2,5-DNT was moderately irritating, producing some necrosis but no edema in the test area. TNT, 2,3-DNT and 3,4-DNT were mildly irritating and the others non-irritating. Some compounds produced less effect than the peanut oil control. A red stain was observed under the TNT patches, and a yellow stain under the 3,4-DNT ones. The colors were similar to the urine colors seen with rodents.

Results of the primary eye irritation studies in rabbits are in Table 4. All compounds were non-irritating.

##### 3. Allergenicity

Results of the guinea pig maximum sensitization test are in Table 5. TNT produced a moderate response to the challenge patch, and 2,6-DNT a mild response. The other compounds had no effects.

## B. Multiple-dose Toxicity<sup>4,5,7/</sup>

Generally speaking, the results with the subacute studies on 2,4-DNT<sup>4/</sup> and subacute studies on 2,6-DNT<sup>5/</sup> were similar. Therefore, discussion will center on 2,4-DNT, with only the different responses of 2,6-DNT noted.

### 1. Critical doses

The critical doses (the no effect dose which caused no apparent effects, the toxic dose which caused some adverse effects without death, and the lethal dose which caused an increase in deaths) for 2,4-DNT are listed in Table 6. 2,6-DNT was substantially similar to 2,4-DNT. The one exception was that similar concentrations of 2,6-DNT had similar effects on both mice and rats, while mice were less affected by 2,4-DNT.

An unusual phenomenon was noted in dogs. The dose accumulated before the onset of neuromuscular toxicity varied little over a wide range of doses. The results are shown in Table 7.

### 2. Target organs

The various toxic effects will be discussed in turn.

#### a. Unlocalized effects

The minimal toxic effect seen, particularly in the chronic studies, was a decrease in weight gain. This was not readily apparent in the dogs, although the high (10 mg/kg/day) and middle (1.5 mg/kg/day) dose dogs were usually the lightest (Figure 3). This effect was very apparent in rats (Figure 4) and mice (Figures 5 and 6), even after only 1 week of feeding.

In the subacute study, almost all the high dose rats (145-266 mg/kg/day) and four of the high dose (25 mg/kg/day) dogs were killed or died before their scheduled termination. In the chronic study, three high dose dogs (10 mg/kg/day) were killed when moribund. The effects in the chronic study in rats were not as dramatic, but half the high dose rats (34-45 mg/kg/day) had died about 4 months earlier than with the controls and other dose groups (19-20 months versus 22-24 months). The high dose mice (900 mg/kg/day) began dying quite early; half died before month 10, about half the life-span of the controls. The unscheduled deaths in dogs were due to the neuromuscular effects; in rats, due to the tumors, non-specific debilitation, or old age; in mice, due to old age, debilitation, and the toxic effects of the high dose of 2,4-DNT.

b. Methemoglobinemia and sequelae

The main specific adverse effect seen in all species was methemoglobinemia (i.e., hemoglobin with the iron oxidized to the ferric state) and its sequelae. Methemoglobin, per se, was rarely seen because the body rapidly removes it from the blood,<sup>10/</sup> and the assay is relatively insensitive. Some animals, especially dogs, occasionally had a blue tint in the skin in the mouth and snout area, indicating transient methemoglobinemia. The most common sequela seen at relatively low doses (such as 10 mg/kg/day in dogs) was Heinz bodies, small crystals of decomposed hemoglobin inside the erythrocytes.

The most frequently observed effect of 2,4-DNT was a "compensated anemia" with Heinz bodies. In the more severely affected animals, the bodies' removing of the large amounts of methemoglobin would produce anemia. Usually, the hematopoietic system could increase production sufficiently to maintain normal erythrocyte levels. However, an increased number of reticulocytes, immature erythrocytes, would occur.

Another effect of methemoglobinemia was widespread deposits of a pigment. This was very common in mice, but also seen in the other species. The pigment appeared as golden-brown to blackish-brown coarse granules, with heaviest deposits in the reticuloendothelial system, liver, spleen and kidneys. Deposits resembled hemosiderin, but gave little or no reaction to Prussian blue, indicating the absence of iron. The chemical identity of the granules is unknown, but they are probably metabolites of 2,4-DNT, with some hemoglobin decomposition products included. Despite its widespread nature, even in some neurons and glial cells, the deposits were apparently benign, with no associated degeneration, necrosis or inflammation.

c. Testicular effect

Males of all species had decreased spermatogenesis. In the most severe instances, there was no spermatogenesis at all and the testes atrophied. The effect was most common in rats given 34 mg/kg/day or more, and complicated the dominant lethal mutation study as discussed below. It affected dogs given 25 mg/kg/day for 13 weeks, but not those given 10 mg/kg/day for as long as 2 years. An analogous effect (lack of corpora lutea) was seen in female mice given 900 mg/kg/day in the chronic study.

d. Neuromuscular effects

The most striking effect in dogs was a group of behavioral changes, indicating a neuromuscular effect. The minimal form was incoordination and stiffness, most apparent in the hind limbs. The

incoordination also affected the tongue and lips, making eating difficult and sometimes causing weight loss. This was compensated for in the later parts of the studies by giving the dogs a soft diet (finely grained feed, moistened to a mushy state). In most cases, these toxic signs regressed within days or weeks. In some cases, the stiffness progressed to a paralysis, usually rigid. The paralysis began in the hind legs, and sometimes regressed, leading to temporary recovery before the toxic signs returned. In a few cases, the paralysis progressed to the forelimbs, then the head and neck. Since the dogs could neither eat nor drink, they were then euthanized. This was the major cause of the unscheduled terminations of the dogs. There was an extremely large range of individual susceptibility. Some dogs would be barely affected whereas others were moribund.

Electrolytes were normal, even in the most affected dogs. When the dogs were euthanized, they relaxed as soon as the barbiturate took effect. No peripheral lesions were seen, but there were central nervous system lesions in the cerebellums of a few of the dogs. It is probable that the mechanism of the effect is central, primarily affecting the cerebellum, which controls coordination of the voluntary muscles. This effect was seen in all dogs given 10 mg/kg/day or more, and in one dog given 1.5 mg/kg/day.

A few rats were slightly affected, having occasional episodes of an unusual gait with widespread hind legs. Somewhat more mice were affected, with an unusual combination of depression with hyperexcitability as well as a hunchbacked posture and stiff-legged gait. These effects, although analogous with those in the dogs, were rarer and had little toxicological importance.

#### e. Liver

There is a well-known progressive development of hepatocellular tumors in rats.<sup>11/</sup> We found this in our chronic study of 2,4-DNT. The first stage is foci and areas of altered hepatocytes. Then, neoplastic nodules develop. Finally, full-fledged hepatocellular carcinomas are found. This was apparent in rats fed 34 to 45 mg/kg/day of 2,4-DNT for 12 months as shown in Table 8. These high dose rats had a greater incidence and severity of alterations. Most of them had nodules, and one already had a carcinoma. As feeding continued, the development continued, as shown in the right portion of Table 8. These data include scheduled and unscheduled terminations of all rats fed 2,4-DNT, including those intended for the ancillary studies, such as metabolism. By the end of the study, the increase in incidence of alterations in middle dose rats (3.9-5.1 mg/kg/day) was statistically significant, although these rats died of old age before their livers developed tumors like the high dose rats.

A hepatocellular dysplasia, seen in some male mice, may be a similar effect. However, this effect was not as dramatic or as well-ordered as that in the rats.

f. Skin tumors

Our high dose rats (34-45 mg/kg/day) on the chronic study also had an increased incidence and earlier development of a variety of externally obvious tumors. Many rats even had multiple tumors, of the same or of differing types. These tumors were a frequent cause of death, due to their diversion of body resources, or to the development of ulcerations of the skin above the tumor, which we used as a criterion for euthanasia. In females, the effect was an increase in mammary fibroadenomas. These tumors are common in rats of this strain, in our experience and in the literature,<sup>12/</sup> but were almost universal in the longer-lived high dose females. In the males, the predominant type was subcutaneous fibromas, affecting almost half of the males surviving over 12 months.

g. Kidney tumors

Mice had a variety of unusual lesions in their kidneys. Both sexes had a dose-related incidence of a "toxic nephropathy" characterized by many cysts lined with cobblestone-like tubular epithelium. Male mice also had atypical epithelium, in the cysts and elsewhere, and in some cases, cystic tumors (Table 9). The tumors were more apparent in the middle dose mice (95 mg/kg/day), because the high dose mice (900 mg/kg/day) did not live long enough to develop them.

h. Reversibility

The only effects which were readily reversed in the 4-week reversibility studies were those on blood. As soon as methemoglobin formation ceased due to withdrawal of 2,4-DNT, the hematopoietic system corrected the anemia.

C. Mutagenesis Studies

1. Ames tests<sup>2/</sup>

The results of the Salmonella/microsome plate tests (Ames tests), described as mutagenic ratios, are given in Table 10. TNT and 2,5-DNT were the most active, being mutagenic in all five tester strains at levels as low as 10 or 30 µg/plate. 2,4-DNT and 3,5-DNT were distinctly less active. 3,5-DNT was active in all strains except TA-1535, but only at levels of 100 to 500 µg/plate. 2,4-DNT was active at 10

$\mu\text{g}/\text{plate}$  in only one strain, and at  $300 \mu\text{g}/\text{plate}$  with another. The remaining compounds tested were less active, since they were mutagenic in only one strain at levels of 300 to 1,000  $\mu\text{g}/\text{plate}$ . Activation with the microsomal fraction had few effects. The only noteworthy ones were a decrease in the toxicity of 2,5-DNT and 3,4-DNT to TA-1535 and a decrease in the mutagenicity of 2,4-DNT to TA-1538.

## 2. Cell cultures<sup>4,5,7/</sup>

We found no mutagenic effects of 2,4-DNT and 2,6-DNT on Chinese hamster ovary cultures. Neither compound had consistent adverse effects on the cytogenetics of cells from dogs and rats fed the compounds for 12 or 24 months.

## 3. Dominant lethal mutation study<sup>7/</sup>

If one finds resorbed implants and/or nonviable fetuses in normal females mated to treated males, one has a dominant lethal mutation effect. This is expressed in Table 11 as the Implant Viability Index. However, 2,4-DNT has a toxic effect on sperm production, as discussed above, which is not a mutagenic effect. This is expressed as the Fertility Index. The first study gave the appearance of a positive result. But the low fertility index, presumably due to the toxic effect, and the few males involved, make the data uncertain. Therefore, we did the later studies in an attempt to learn if there is a dose low enough to avoid the antisperm effect but high enough to produce the looked-for antifertilization effect.

As shown in Table 11, the doses in the second study were too low, those in the third study too high. Finally, after the fourth study, we concluded that 2,4-DNT does not have a dominant lethal mutation effect because the sperm, if present, produced normal, and viable implants. It was noted that many treated males mated, but the vaginal plugs contained no sperm detectable in the smears.

## D. Three Generation Reproduction Study<sup>7/</sup>

This study generated considerable data. Adverse effects such as fewer pups were seen in only the high dose rats ( $34-45 \text{ mg/kg/day}$  in the chronic toxicity study). These effects were due to a number of factors, including maternal neglect, maternal death during parturition, and spermless vaginal plugs. In addition, the sires, dams and pups had low body weight and a generally poor appearance. We concluded that 2,4-DNT had no specific reproductive effects, except that on spermatogenesis, and that the poor reproductive performance was due to the general toxicity of 2,4-DNT and the resulting poor condition of the dams.

## E. Metabolism Studies

### 1. In vivo studies

#### a. Single-dose<sup>1,2/</sup>

The disposition of <sup>14</sup>C-containing products from rats given doses (about one-tenth the LD<sub>50</sub>) of <sup>14</sup>C-ring-labeled-TNT, -DNTs, or -4-ADNT is shown in Figure 7. Compounds were well absorbed (at least 50 to 75% of the dose) and rapidly excreted in the urine. Negligible amount of radioactivity was expired as CO<sub>2</sub>. The radioactivity was widely distributed within the carcass, and concentrated in the liver and kidney. Thin-layer chromatography of the urine found little, if any of the parent compound, and a number of metabolites.

#### b. Biliary excretion<sup>4/</sup>

The biliary excretion of the compounds was studied in rats. From 10 to 28% of an oral dose was excreted in the bile (Figure 8), so the total absorption is probably greater than that indicated from the carcass plus urine radioactivity.

#### c. 2,4-DNT metabolism<sup>4/</sup>

The urinary compounds derived from 2,4-DNT were studied in detail. There are two primary metabolic reactions: reduction of one or both nitros to aminos and oxidation of the side-chain through benzyl alcohol and benzaldehyde to benzoic acid. Thus, there are 16 possible metabolic products (Figure 9). The benzaldehydes (compound IX through XII) would react with any available amine group to form Schiff bases; these products probably accounted for some of the unidentified radioactivity. The major compounds were the benzyl alcohols V, VI and VII. We found varying lesser amounts of compounds I (2,4-DNT), II, III, IV, VIII and XIII. We lacked standards to identify compounds XIV through XVI. Most of the excreted compounds had undergone secondary metabolism (glucuronidation, sulfation) before excretion.

#### d. 2,4-DNT in various species<sup>4/</sup>

Parallel experiments were done with 2,4-DNT in mice, rabbits, dogs and rhesus monkeys. Mice differed considerably from the other species, because they absorbed much less (Figure 10). This poor absorption occurred in two strains, albino CD-1® and dark B6C3F1. This is apparently the cause for the lesser toxicity of 2,4-DNT in mice, noted above. However, once absorbed, the 2,4-DNT was treated similarly by all species, giving similar tissue distributions, primary metabolism products (Figure 9) and secondary conjugation products (Figure 11).

e. 2,4-DNT metabolism after chronic feeding<sup>7/</sup>

Feeding rats 2,4-DNT for 3, 9 or 20 months did not affect how they handled a single oral dose of <sup>14</sup>C-2,4-DNT. The results were substantially the same as those described above.

2. In vitro metabolism<sup>4/</sup>

2,4-DNT was incubated with liver preparations from five species: rat, mouse, rabbit, dog and monkey. Under aerobic conditions, the primary product was 2,4-dinitrobenzyl alcohol and the secondary, amino-nitrotoluenes. Under anaerobic conditions, the relative proportions of these two main products were reversed. We were unable to obtain human liver specimens for a direct comparison. However, the similarity between the five mammals of four orders studied makes it probable that humans would be substantially similar.

3. Interactions<sup>4,5/</sup>

In experiments with rats, pretreatment with 2,4-DNT did not affect the duration of zoxazolamine-induced paralysis or the hepatic nitroanisole O-methylase activity. Pretreatment with 2,6-DNT did decrease the duration of paralysis and increase the enzyme activity. It is not known why 2,6-DNT affected the drug-metabolizing enzyme systems involved in these tests and why 2,4-DNT did not.

#### IV. CONCLUSIONS

##### A. Acute Toxicity

These conclusions apply to all compounds listed: 2,4,6-TNT, the DNT isomers, and the two ADNT isomers tested.

1. The tested compounds have generally similar toxicity, quantitatively and qualitatively.
2. Two toxic mechanisms were apparent. The lethal one was central nervous system depression. Some compounds (TNT, 3,4-DNT, 2-ADNT, 4-ADNT) also had a lesser effect of central nervous system excitability, apparent in the form of convulsions or hyperreflexia.
3. Colored urine provided evidence of significant biotransformation.
4. These compounds had relatively minor effects as skin and eye irritants.
5. Although all these compounds are presumably capable of causing allergic reactions via reduction to an aromatic amine which could act as a hapten, only two showed only mild or moderate allergenicity.

##### B. Multiple Dose Toxicity

These data are for 2,4-DNT. Except for the one stated difference, it is expected that the other nitrotoluenes are similar.

1. In sufficient dose, 2,4-DNT is toxic; in sufficient dose over a sufficient time, 2,4-DNT is carcinogenic.
2. The no-effect levels for 2,4-DNT are 0.2 mg/kg/day for 2 years dosing in dogs, 0.57 and 0.71 mg/kg/day for lifetime feeding in male and female rats, respectively, and 13.5 mg/kg/day for lifetime feeding in mice. With the other compounds, the no-effect levels for mice would be significantly lower, comparable to those of the rats, because the LD<sub>50</sub>s for mice are closer to those for rats, unlike 2,4-DNT.
3. The effective levels for the toxic and carcinogenic effects were similar in rats and mice. No carcinogenicity was observed in dogs, presumably, due to the relatively limited dosing period.
4. The most pervasive adverse effect was methemoglobinemia and sequelae. Mechanisms appear to be those already well known.

5. Heinz bodies, one of the consequences of methemoglobinemia, appear to be the most useful indicator of exposure to toxic levels of 2,4-DNT. In severe cases, reticulocytosis is a convenient indicator.

6. A decrease in spermatogenesis was seen in all species. With dogs there was a distinct threshold between 10 and 25 mg/kg/day. This effect was seen only at doses producing severe toxicity of other types.

7. A neuromuscular effect was seen, especially in dogs. The mechanism is central, probably involving a biochemical lesion which affects the cerebellum most strongly.

8. The liver was a target organ in rats, who developed the classical progression to hepatocellular carcinoma. The molecular mechanism may be related to the toxic jaundice observed in some human fatalities from TNT.

9. Rats had an increased incidence of "background tumors," particularly fibromas in males and mammary fibroadenomas in females.

10. The kidney was a target organ in mice, who had cystic alterations which developed into tumors of various types.

#### C. Special Toxicities

##### 1. Mutagenicity

a. These compounds, especially TNT, were positive in the Ames test without activation.

b. There was no unequivocal evidence of mutagenicity of 2,4-DNT and 2,6-DNT in various mammalian test systems.

##### 2. Reproductive toxicity

2,4-DNT showed no evidence of teratology or any other specific reproductive effect, except causing aspermatogenesis.

#### D. Metabolism

These data apply to all these compounds, with the stated exception.

1. The compounds are well absorbed after oral dosing of mammals, widely distributed but concentrated only in the liver and kidney, extensively metabolized in the liver and excreted in the urine and, to a lesser extent, the bile.

2. The only exception is 2,4-DNT which is poorly absorbed by mice. However, the absorbed portion of the dose is handled similarly to that absorbed by other species.

3. Primary metabolism is in the liver by one or both of two pathways: oxidation of the methyl through benzyl alcohol and benzaldehyde to a benzoic acid, and reduction of nitros to amines.

4. Secondary metabolism is primarily glucuronidation with some sulfation and some excretion of unconjugated metabolites.

5. Human metabolism is probably similar to that of the other mammals.

6. Data on interactions with drug metabolizing enzymes by 2,4-DNT and 2,6-DNT are contradictory; therefore, no general conclusions can be drawn.

#### E. Water Quality Criteria

Because 2,4-DNT has carcinogenic effects, an ambient water concentration of zero is necessary for maximum protection of human health. However, using EPA developed methodology, exposure to 1.152 µg/liter of 2,4-DNT for a lifetime produces an estimated risk of  $10^{-5}$  (1 in 100,000) that a tumor will develop in man. A tenfold decrease in dose would produce a tenfold decrease in the estimated risk. Because of the similarities between the isomeric DNT's, this limit for 2,4-DNT is appropriate for a normal mixture of DNT's.

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**TABLE I**  
**AUTE ORAL TOXICITIES (mg/kg) OF VARIOUS NITROTOLUENE COMPOUNDS IN**  
**MALE AND FEMALE RATS**

Compounds	Males			Females		
	LD <sub>50</sub> ± S.E.	95% Confidence Limits	Slope ± S.E.	LD <sub>50</sub> ± S.E.	95% Confidence Limits	Slope ± S.E.
TNT	1,010 ± 41	922 - 1,108	5.96 ± 1.50	820 ± 32	747 - 889	5.68 ± 1.41
2,3-DNT	1,102 ± 20	1,011 - 1,169	15.46 ± 6.84	911 ± 65	584 - 1,049	3.25 ± 1.37
2,4-DNT	568 ± 59	434 - 705	2.25 ± 0.61	650 ± 49	520 - 743	3.53 ± 0.94
2,5-DNT	616 ± 34	532 - 707	4.20 ± 1.37	517 ± 25	477 - 575	5.56 ± 1.85
2,6-DNT	535 ± 58	397 - 646	1.79 ± 0.41	795 ± 22	744 - 844	8.48 ± 2.14
3,4-DNT	907 ± 42	815 - 1,011	4.78 ± 1.28	807 ± 33	721 - 874	4.67 ± 1.23
3,5-DNT	309 ± 13	278 - 340	5.03 ± 1.48	216 ± 19	160 - 256	3.44 ± 1.11
2-ADNT	2,240 ± 85	2,070 - 2,430	8.97 ± 3.41	1,394 ± 191	989 - 2,830	1.85 ± 0.79
4-ADNT	1,360 ± 53	1,260 - 1,465	18.17 ± 2.34	959 ± 76	787 - 1,154	3.60 ± 1.07

TABLE 2

ACUTE ORAL TOXICITIES (mg/kg) OF VARIOUS NITROTOLUENE  
COMPOUNDS IN MALE AND FEMALE MICE

Compounds	Males			Females		
	LD <sub>50</sub> ± S.E.	95% Confidence Limits	Slope ± S.E.	LD <sub>50</sub> ± S.E.	95% Confidence Limits	Slope ± S.E.
TNT	1,014 ± 52	905 - 1,163	3.47 ± 1.06	1,009 ± 54	880 - 1,117	3.86 ± 0.88
2,3-DNT	1,372 ± 34	1,285 - 1,441	8.53 ± 2.26	1,089 ± 32	1,029 - 1,175	7.96 ± 2.03
2,4-DNT	1,954 ± 68	1,848 - 2,178	4.50 ± 1.15	1,340 ± 67	1,205 - 1,500	4.15 ± 0.93
2,5-DNT	652 ± 28	585 - 712	5.05 ± 1.29	659 ± 12	633 - 690	12.97 ± 3.51
2,6-DNT	621 ± 51	488 - 721	3.25 ± 0.87	807 ± 35	725 - 893	5.93 ± 1.51
3,4-DNT	855 ± 37	787 - 958	4.19 ± 1.10	747 ± 26	702 - 821	5.74 ± 1.28
3,5-DNT	611 ± 43	523 - 714	2.90 ± 0.70	607 ± 21	559 - 650	5.95 ± 1.45
2-ADNT	1,722 ± 154	1,450 - 2,131	2.90 ± 0.42	1,522 ± 71	1,372 - 1,692	5.93 ± 1.50
4-ADNT	1,342 ± 107	1,141 - 1,611	2.32 ± 0.49	1,495 ± 90	1,318 - 1,713	3.69 ± 0.81

TABLE 3

PRIMARY SKIN IRRITATION OF VARIOUS NITROTOLUENE  
COMPOUNDS IN RABBITS

<u>Compounds</u>	<u>Primary Irritation Score<sup>a/</sup></u>
TNT	1.0 <sup>b/</sup>
2,3-DNT	1.78
2,4-DNT	0.25 <sup>c/</sup>
2,5-DNT	3.80
2,6-DNT	0.21 <sup>d/</sup>
3,4-DNT	2.00
3,5-DNT	< 0.2
2-ADNT	0.21
4-ADNT	< 0.2
Peanut Oil (vehicle control)	0.33

a/ Average value of six rabbits with intact and abraded skin in each test group. The compounds are classified as follows:

- > 0.2 over controls is mild irritant
- > 2.5 over controls is moderate irritant
- > 5.0 over controls is severe irritant

b/ Red color under all patches at 24 hours.

c/ No edema was apparent but the entire area covered by the compound was undergoing necrosis in 24 hours in both the intact and abraded skin.

d/ Yellow color under all patches at 24 hours.

TABLE 4

PRIMARY EYE IRRITATION OF VARIOUS NITROTOLUENE  
COMPOUNDS IN RABBITS

<u>Compounds</u>	<u>Results<sup>a/</sup></u>
TNT <sup>b/</sup>	Nonirritant
2,3-DNT	Nonirritant
2,4-DNT	Nonirritant
2,5-DNT	Nonirritant
2,6-DNT	Nonirritant
3,4-DNT <sup>c/</sup>	Nonirritant
3,5-DNT	Nonirritant
2-ADNT	Nonirritant
4-ADNT	Nonirritant
Peanut Oil (vehicle control)	Nonirritant

a/ Six rabbits per test group.

b/ Red color around the eye at 24 hours.

c/ Yellow color around the eye at 24 hours.

TABLE 5  
DERMAL SENSITIZATION OF VARIOUS NITROTOLUENE COMPOUNDS  
IN GUINEA PIGS

<u>Compound</u>	<u>Number Responding</u>	<u>% Response</u>	<u>Sensitization</u>
TNT	4/10	40%	moderate
2,3-DNT	0/10	0	none
2,4-DNT	0/10	0	none
2,5-DNT	0/10	0	none
2,6-DNT	2/10	20%	mild
3,4-DNT	0/10	0	none
3,5-DNT	0/10	0	none
2-ADNT	0/10	0	none
4-ADNT	0/10	0	none

TABLE 6

CRITICAL ORAL DOSES OF 2,4-DNT IN SUBACUTE AND  
CHRONIC STUDIES OF DOGS, RATS AND MICE

		mg/kg/day	
	<u>No Effect</u>	<u>Toxic</u>	<u>Lethal</u>
<b>Dogs (gavage)</b>			
13 weeks	5	-	25
2 years	0.2	1.5	10
<b>Rats (in feed)</b>			
13 weeks	0.07%	0.2%	0.7%
	M - 34	M - 93	M - 266
	F - 38	F - 108	F - 145
2 years	0.0015%	0.01%	0.07%
	M - 0.57	M - 3.9	M - 34
	F - 0.71	F - 5.1	F - 45
<b>Mice (in feed)</b>			
13 weeks	0.2%	0.7%	-
	M - 137	M - 413	
	F - 147	F - 468	
2 years	13.5 (0.01%)	95 (0.07%)	900 (0.5%)

TABLE 7

ACCUMULATED ORAL DOSE OF 2,4-DNT TO CAUSE  
NEUROMUSCULAR SYMPTOMS IN DOGS

DAILY DOSE (mg/kg)	DAYS TO CAUSE TOXICITY	ACCUMULATED DOSE (mg/kg)
250	1	250
50	7	350
30	13	390
25	10-14	250-350
10	50 +	500 +
1.5	465	700

TABLE 8

## INCIDENCE OF HEPATOCELLULAR LESIONS IN RATS FED 2,4-DNT

Lesion	Dose (%) in feed):	Fed 12 months			Fed over 12 months				
		0	0.0015	0.01	0.07	0	0.0015	0.01	0.07
Alterations, + or +	3/15 <sup>a/</sup>	5/16	6/16	1/15		16/48	28/63	28/46	29/63
Alterations, ++ or +++	0/15	0/16	1/16	10/15					
Neoplastic Nodules	5/15	1/16	0/16	13/15	1/48	5/63	3/46	8/63	
Hepatocellular carcinomas	6/15	0/16	0/16	1/15	1/48	0/63	2/46	24/63	

a/ Number of rats with lesion/number of rats with readable tissues.

TABLE 9

INCIDENCE OF RENAL TUMORS IN MICE FED 2,4-DNT

<u>Dose (% in feed)</u>	<u>Fed 12 Months</u>		<u>Fed 24 Months<sup>a/</sup></u>	
	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>
0	0/8	0/8	0/25	0/23
0.01	0/8	0/8	6/25	0/20
0.07	0/8	0/8	16/21	0/23
0.5	2/8	0/8	10/32	1/24

a/ Including mice dying at unscheduled times.

TABLE 10

MUTAGENICITY OF NUNITIONS COMPOUNDS USING THE  
SALMONELLA/MICROSOME PLATE TEST

Test Compound	<u>ug/b/</u>	Mutagenic Ratio <sup>a/</sup>											
		TA-100		TA-1535		TA-98		TA-1537		TA-1538			
		Ic/	IId/	I	II	I	II	I	II	I	II	I	II
TNT	10	1.5	1.5	1.0	1.1	14.8*	3.6*	4.3*	2.0*	26.0*	2.3*		
	30	2.6*	2.3*	0.9	1.4	17.9*	18.2*	5.2*	4.0*	44.8*	9.3*		
	100	4.6*	2.6*	1.3	1.3	7.9*	4.8*	6.5*	3.2*	34.5*	21.9*		
	300	T*	7.9*	0.8	2.4*	11.3*	7.2*	T	9.0*	17.8*	13.2*		
	1,000	T	T	T	T	T	12.5*	T	T	T	11.5*		
1,2-DNT	30	1.0	1.0	0.8	0.8	0.8	1.2	0.7	1.3	1.1	1.3		
	100	0.9	1.4	0.6	0.6	1.0	1.3	1.0	1.0	0.9	1.0		
	300	1.6	1.0	0.5	1.0	1.1	0.7	1.2	0.9	1.9	1.2		
	1,000	T	T	T	0.9	T	1.0	0.3	1.3	0.1	2.8*		
1,4-DNT	10	0.8	0.9	1.4	1.2	1.0	0.9	1.2	0.8	2.0*	0.8		
	30	0.9	1.2	1.0	0.9	1.0	1.1	0.9	0.8	3.0*	1.3		
	100	1.0	1.1	0.3	0.6	1.5	1.8	0.9	1.2	6.6*	1.3		
	300	2.0*	2.2*	1.1	1.3	1.2	1.8	1.6	1.4	13.5*	1.2		
	1,000	2.8*	4.2*	0.5	1.6	0.8	1.3	T	1.8	T	4.3*		
1,5-DNT	30	1.1	1.2	1.5	1.0	1.6	1.6	0.6	1.7	3.6*	2.3*		
	100	1.4	1.3	0.9	1.6	2.0*	2.2*	2.6*	2.9*	13.2*	5.5*		
	300	2.1*	2.5*	T	1.8	2.8*	3.4*	1.0	2.4*	7.3*	14.3*		
	600	T	3.2*	T	3.7*	T	2.2*	2.8*	2.8*	T	17.5*		
	1,000	T	5.7*	T	T	T	1.9	T	T	T	T		
1,6-DNT	30	1.3	1.2	0.8	1.1	1.4	1.4	1.1	0.8	1.6	0.9		
	100	1.2	1.1	0.9	0.8	1.2	1.1	1.3	1.0	1.4	1.0		
	300	1.7	1.6	1.0	1.2	1.2	1.1	1.2	0.6	2.7*	0.5		
	1,000	1.6	1.3	1.3	1.3	1.0	1.5	0.6	1.8	4.9*	1.0		
3,4-DNT	10	1.1	0.9	1.4	1.0	1.0	1.0	0.9	0.5	0.8	0.9		
	100	1.1	1.1	T	0.6	0.8	0.9	1.4	0.8	1.0	1.0		
	300	1.4	1.6	T	0.7	1.0	0.6	0.6	0.3	1.3	1.0		
	1,000	2.6*	4.6*	T	0.3	0.4	0.9	T	0.8	0.2	1.7		
3,5-DNT	10	1.4	1.0	1.4	0.6	1.0	0.6	1.1	1.2	1.6	0.9		
	100	1.1	0.9	1.5	0.7	1.2	1.1	1.8	1.2	5.0*	1.8		
	500	3.0*	1.9	1.1	1.3	7.7*	3.4*	2.3*	2.3*	29.2*	14.4*		
	1,000	1.4*	5.3*	T	1.1	4.7*	8.0*	T	5.9*	T	52.1*		

a/ Mutagenic Ratio: number of histidine revertants in these cultures/number of histidine revertants in the control dish.

b/ ug of test compound/plate introduced into 3.0 ml of top agar.

c/ Test run without metabolic activation.

d/ Test run with metabolic activation.

e/ T = microbial toxicity.

\* Classified as mutagenic (M.R.  $\geq$  2.0).

TABLE 11

RESULTS OF THE DOMINANT LETHAL MUTATION STUDIES

Dose (# 2,4-DNT in Feed for 13 Weeks)	First Study		Second Study		Third Study		Fourth Study	
	<u>PIa/</u>	<u>VIb/</u>	<u>PII</u>	<u>VI</u>	<u>PI</u>	<u>VI</u>	<u>PI</u>	<u>VI</u>
0.5								
0.2	20 ± 13(5)c,d/	0d/			e/			
0.15					f/			
0.10								
0.07								
0.02	67 ± 14(4)	62 ± 24	93 ± 7(10)	94 ± 1				
0.01			100 ± 0(7)	97 ± 1				
0.0015			89 ± 6(9)	94 ± 1				
0	92 ± 8(4)	92 ± 1	96 ± 4(8)	96 ± 1	91 ± 6(21)	96 ± 1		

a/ Fertility index = confirmed pregnancies/plug positive females × 100.

b/ Implant viability index = viable fetuses/implants × 100.

c/ Mean ± standard error (number of males).

d/ Significantly different from control.

e/ None of the three survivors of 15 treated males mated.

f/ No pregnancies.

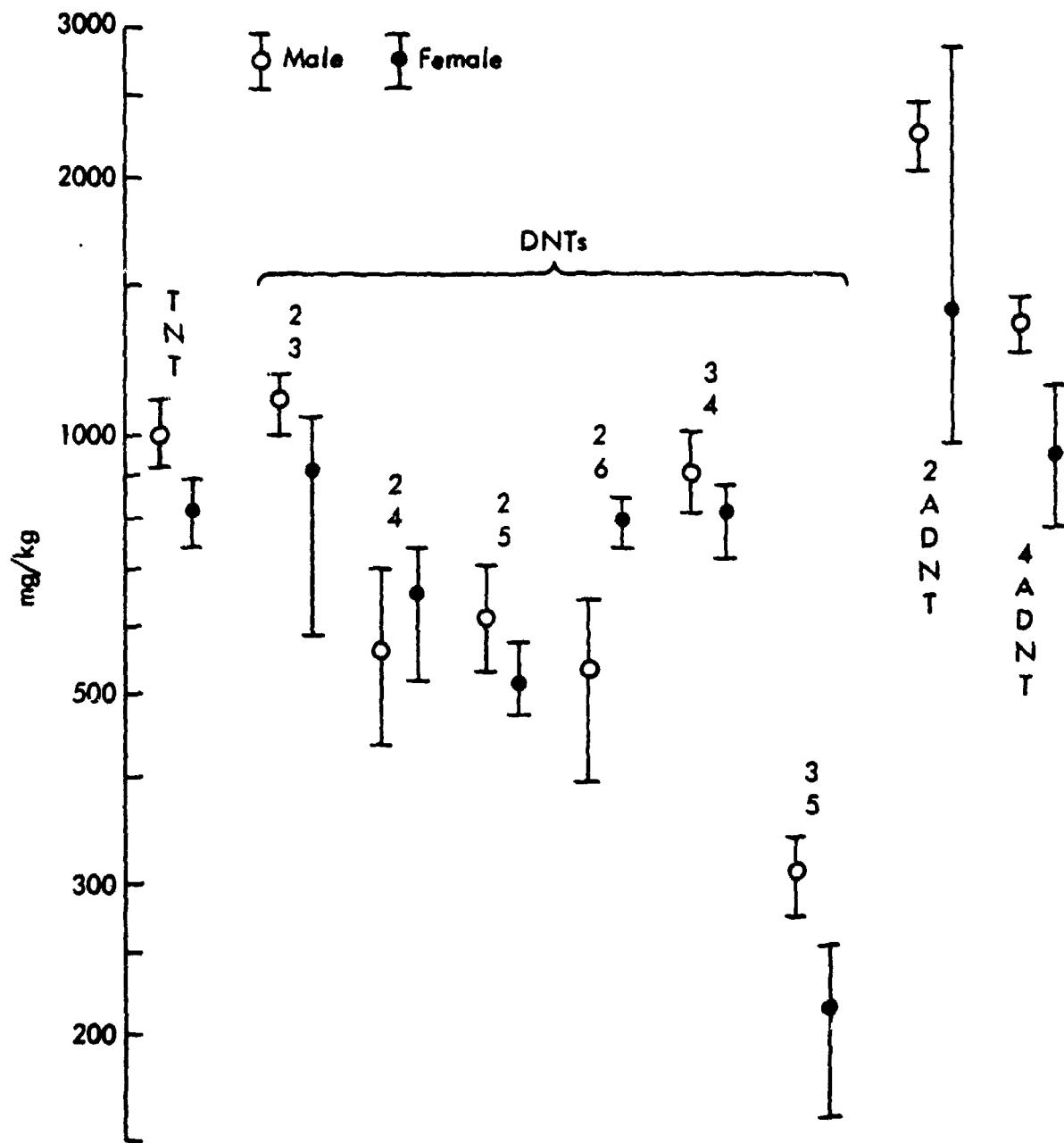


Figure 1 - Oral LD<sub>50</sub>s and their standard errors in rats.

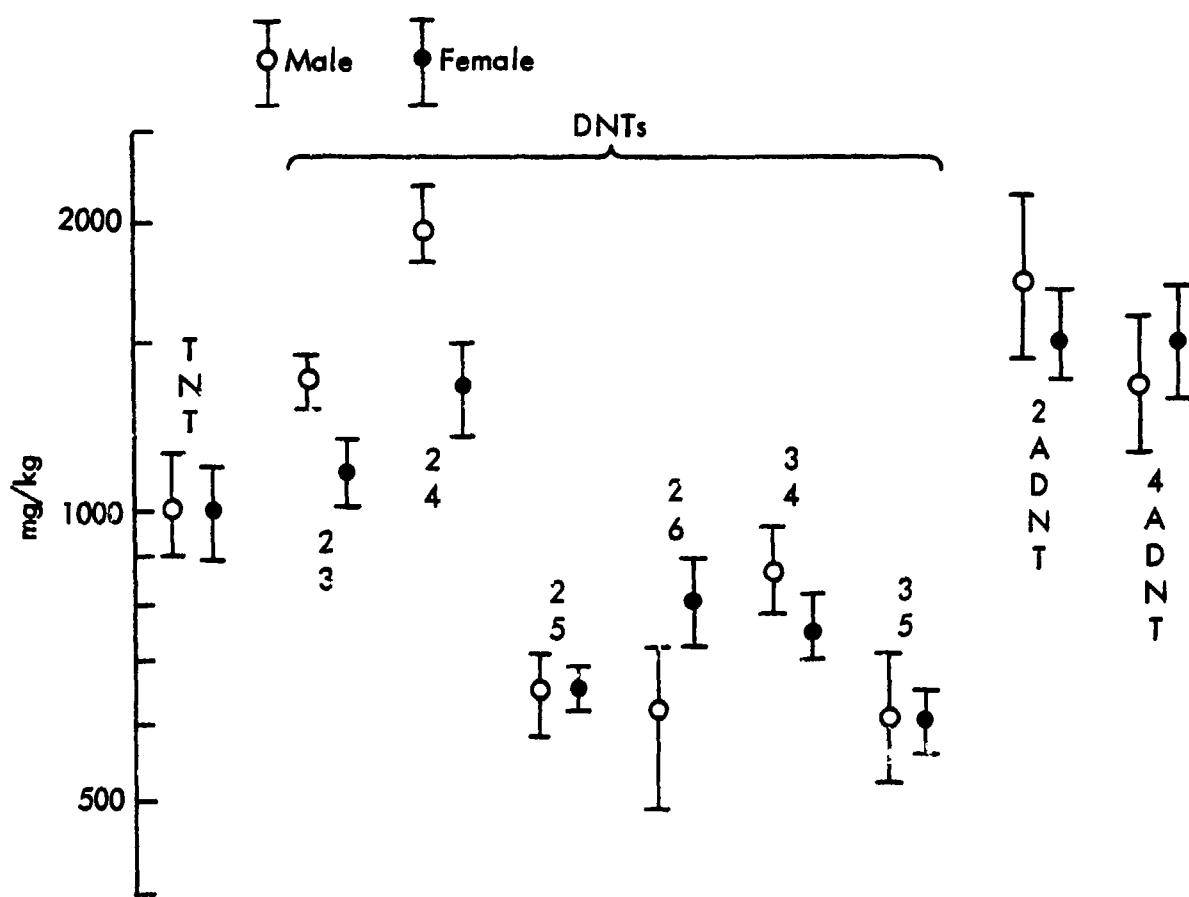
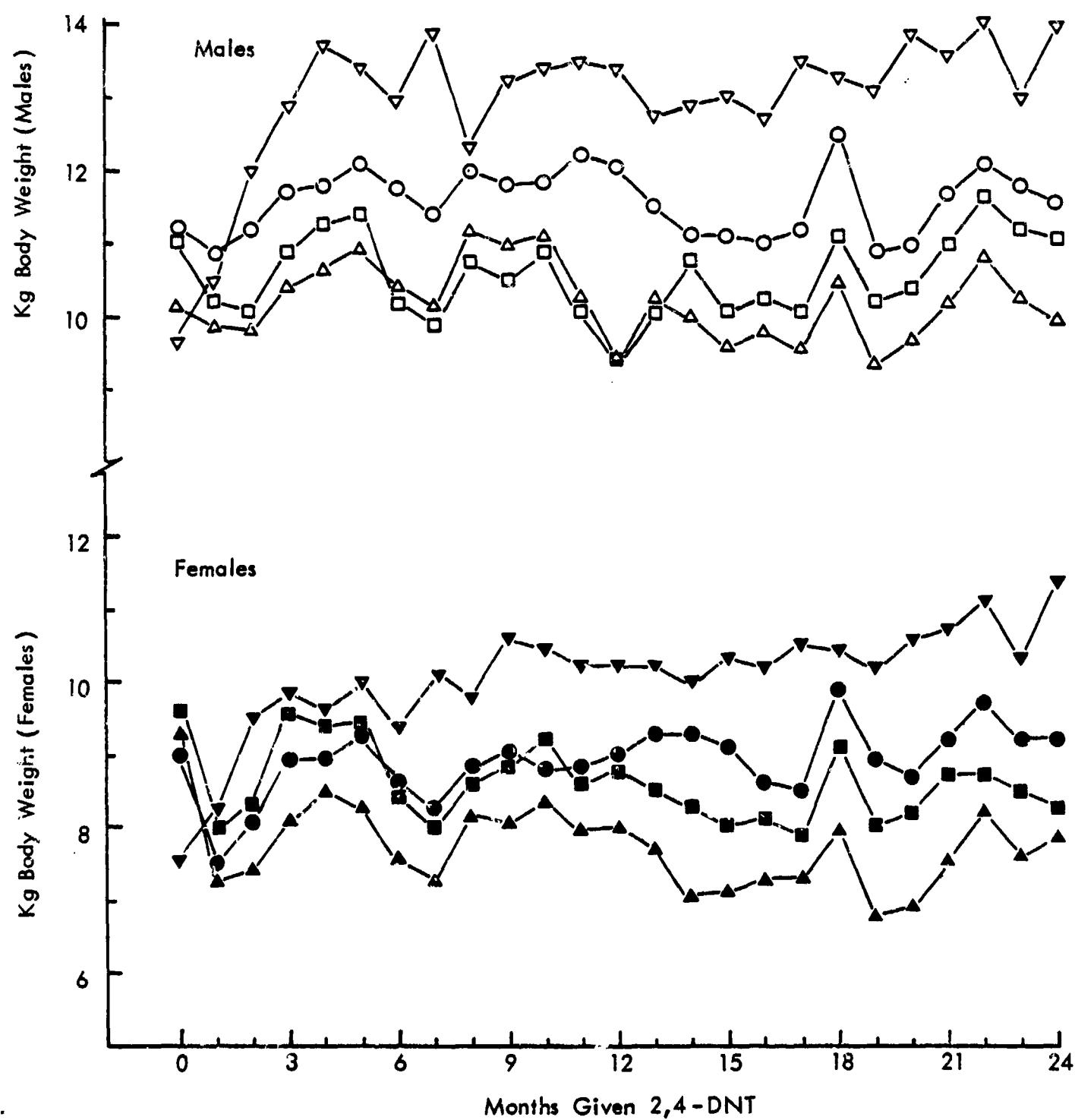


Figure 2 - Oral LD<sub>50</sub>s and their standard errors in mice.



Dose:

- Control
- ▽ 0.2 mg/kg/Day
- △ 1.5 mg/kg/Day
- 10.0 mg/kg/Day

Figure 3 - Average Body Weights of Dogs Given 2,4-DNT

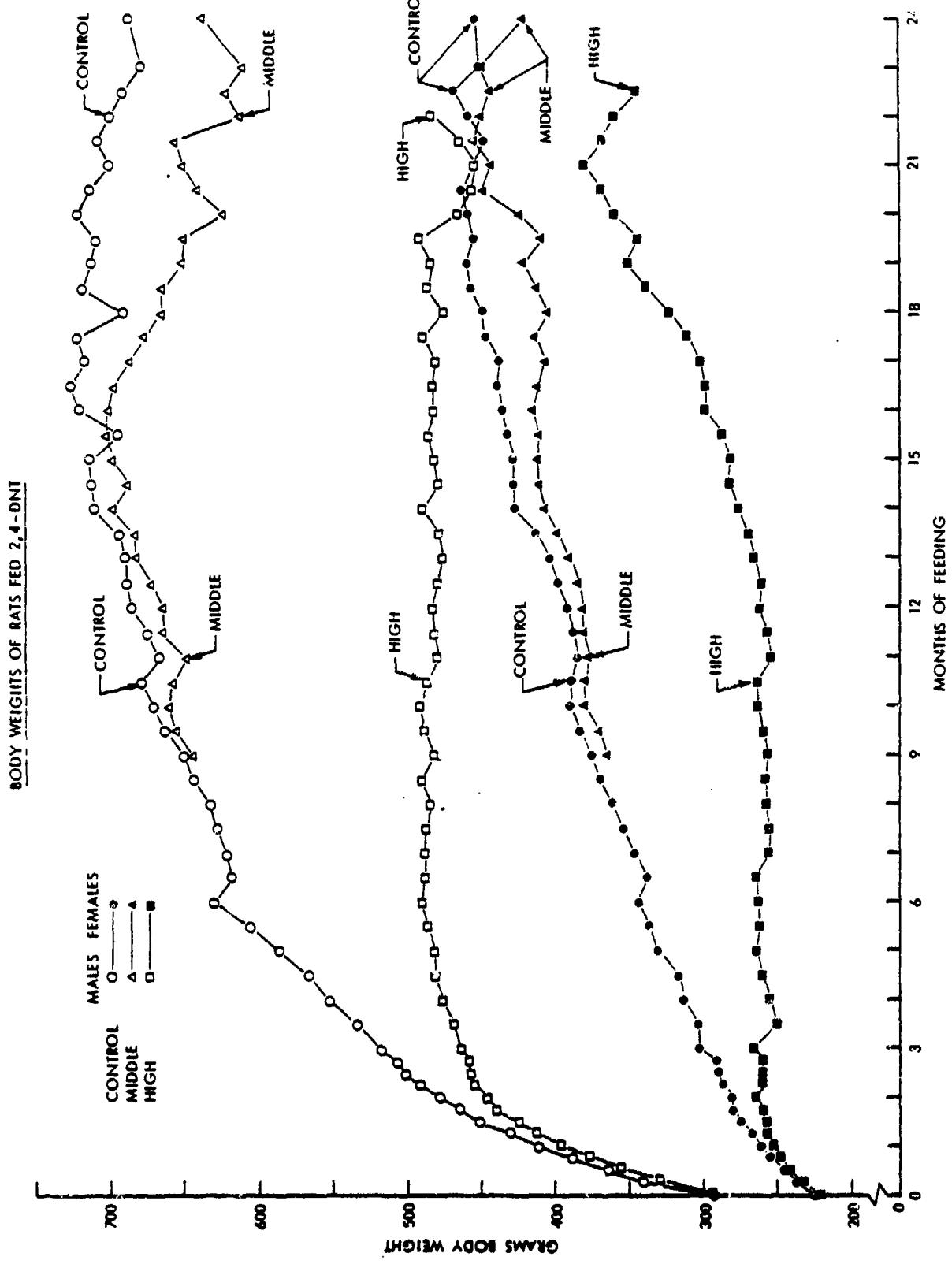


Figure 4 - Body Weights of Rats Fed Various Doses of 2,4-DNT

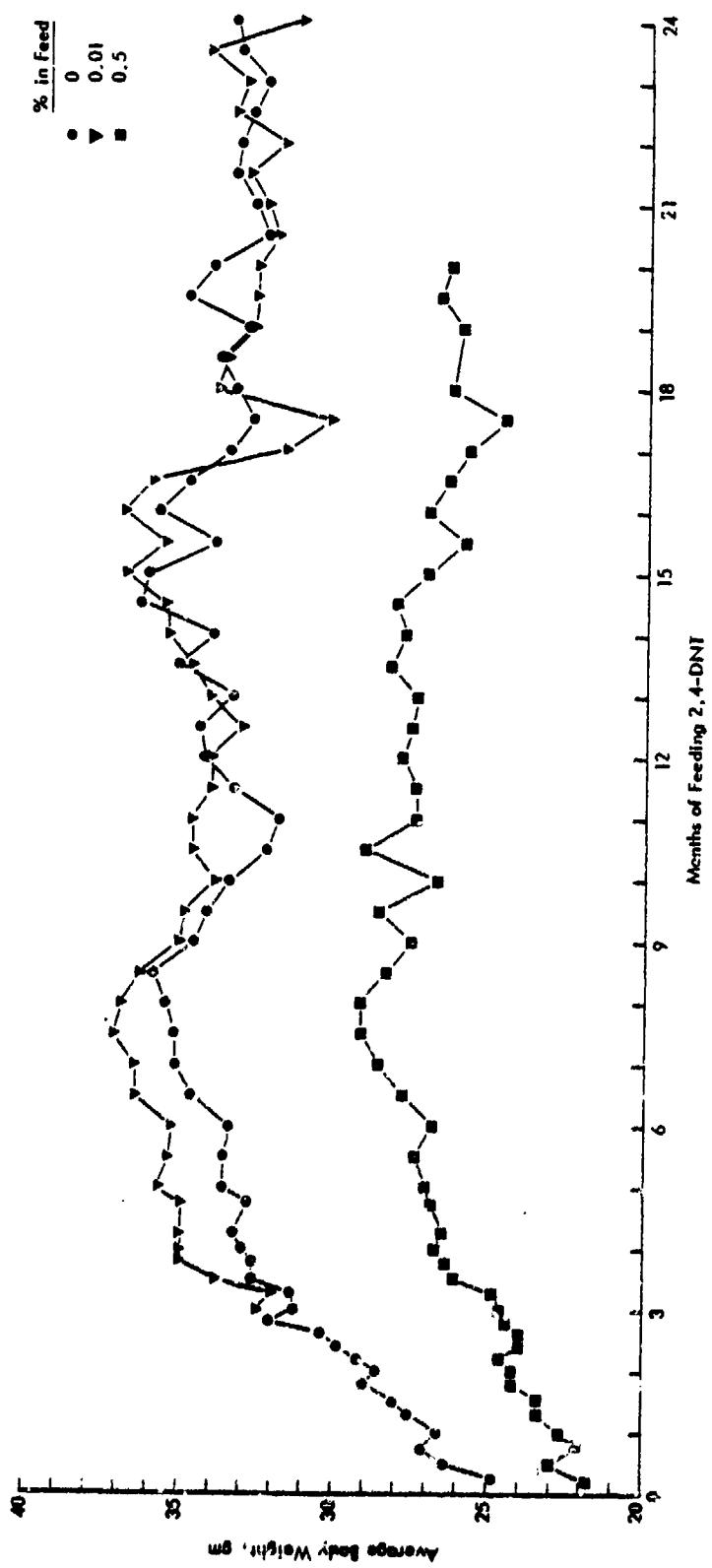


Figure 5 - Average Body Weights of Female Mice Fed 2,4-DNT

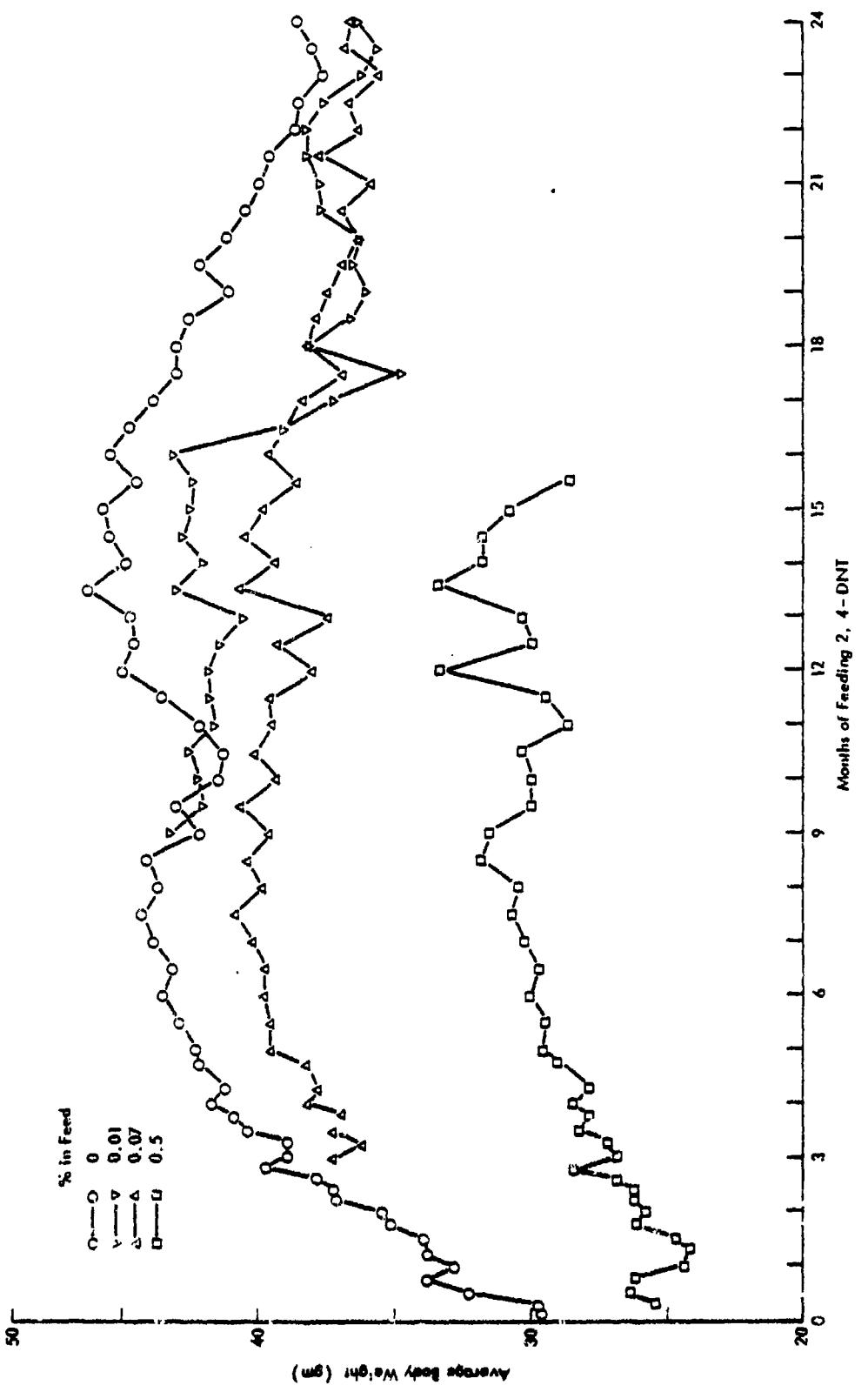


Figure 6 - Average Body Weights of Male Mice Fed 2,4-DNT

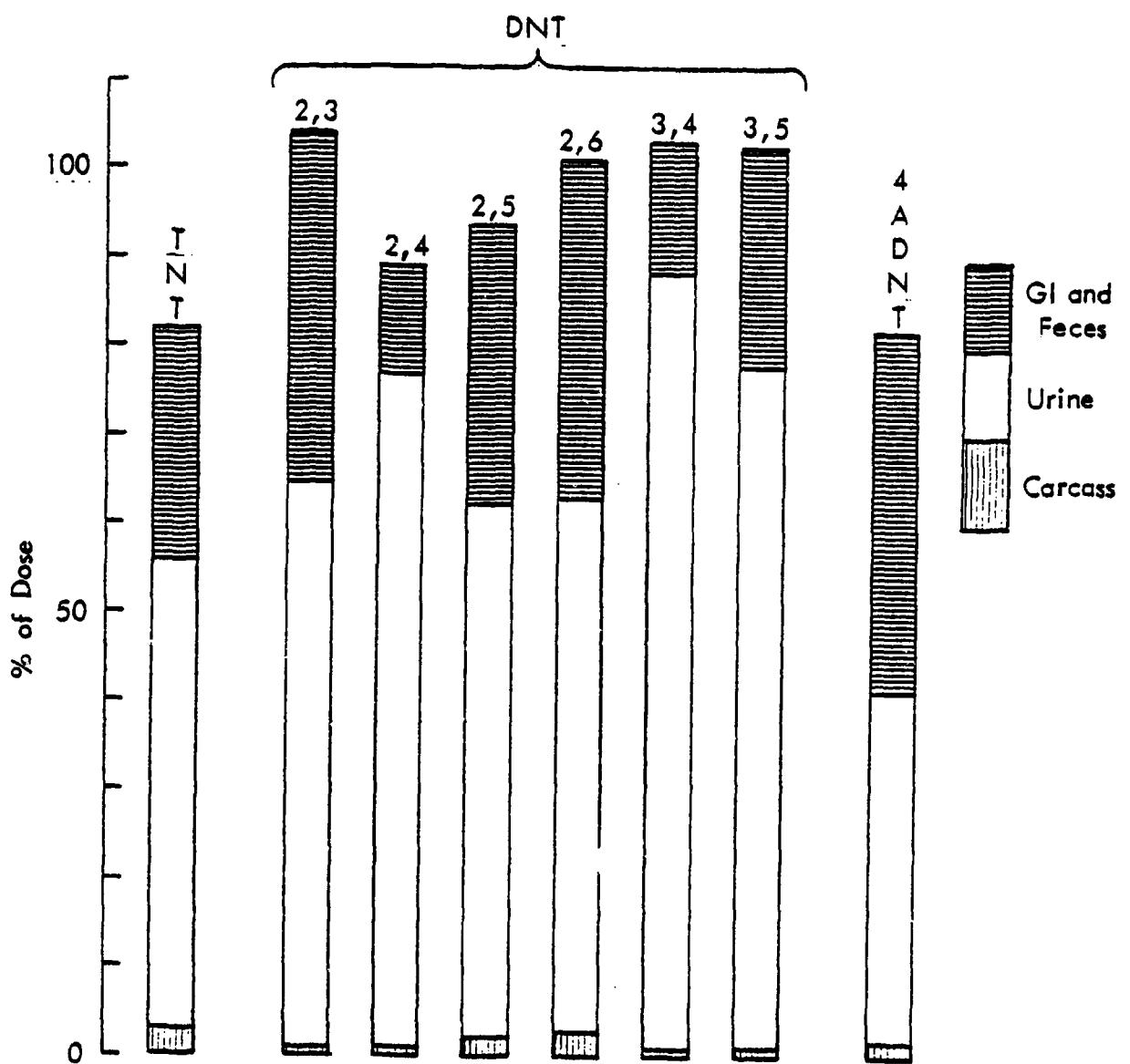


Figure 7 - Disposition in Female Rats of Radioactivity from Ring-<sup>14</sup>C-Nitrotoluenes 24 hr After Oral Dosing

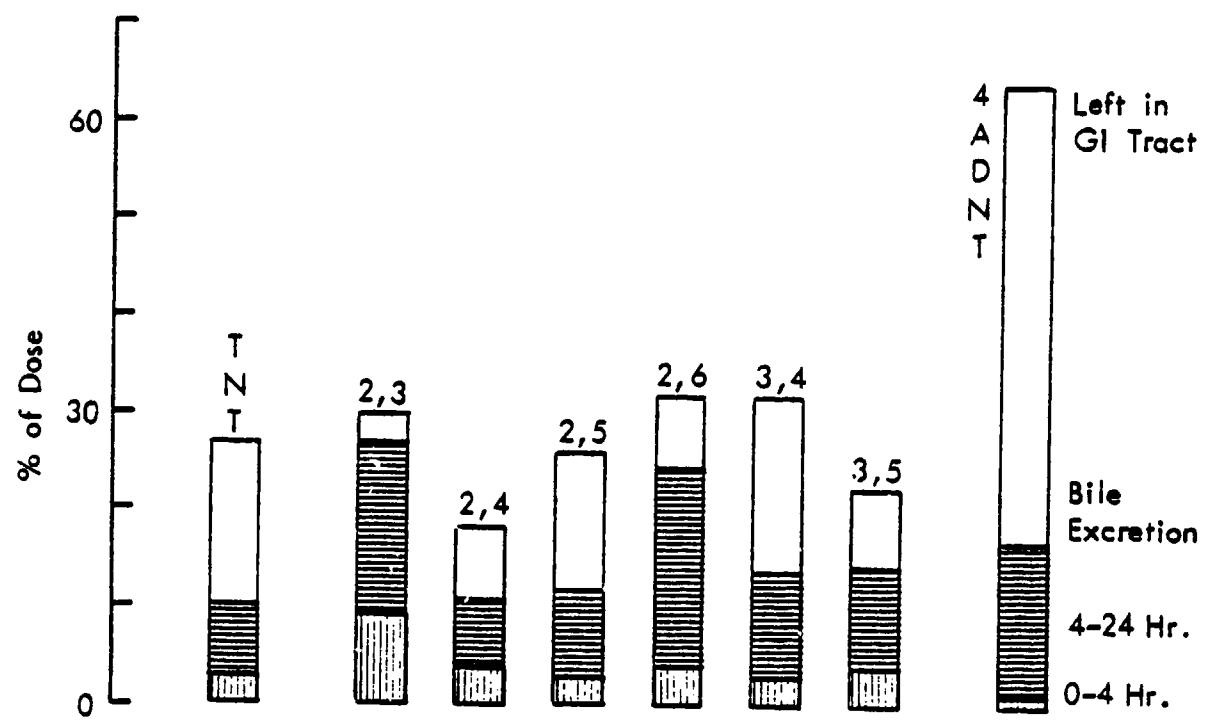


Figure 8 - Biliary Excretion in Female Rats of Nitrotoluenes  
24 hr After an Oral Dose

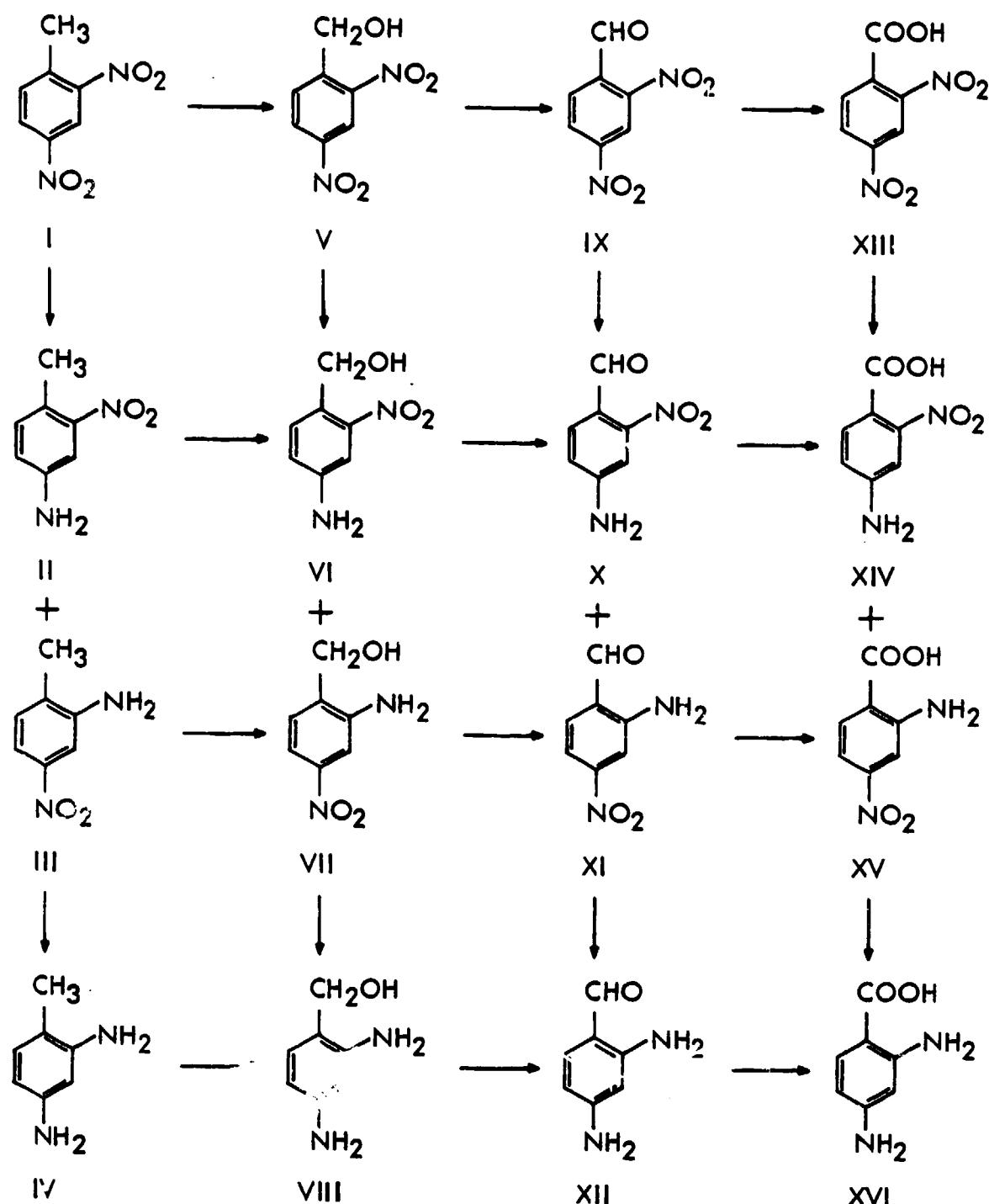


Figure 9 - Possible Early Steps (Phase I) of Metabolic Pathways of 2,4-DNT in Mammals

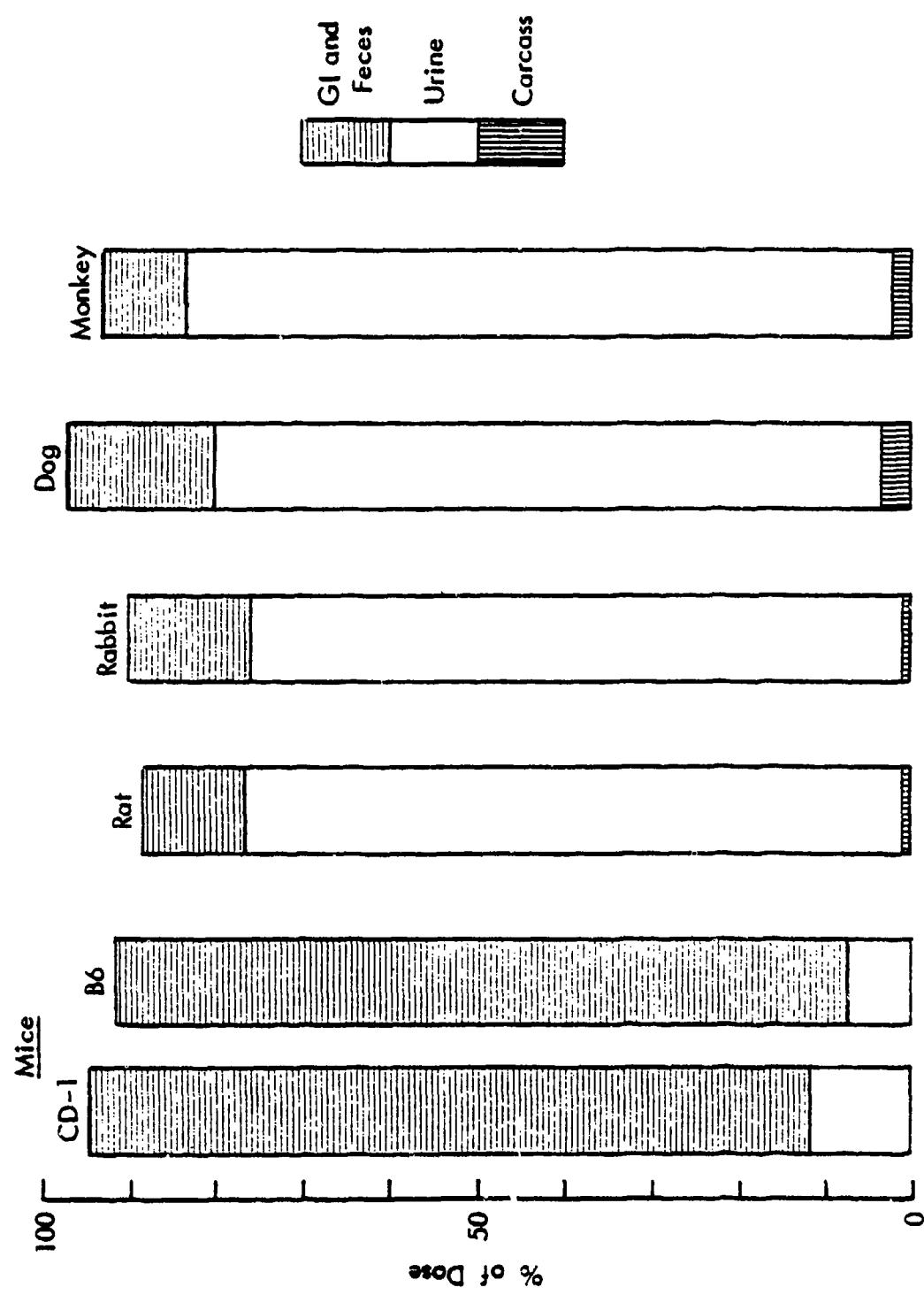


Figure 10 - Disposition of Radioactivity from Ring-14C-2,4DNT 24 hr After Oral Dosing of Various Species

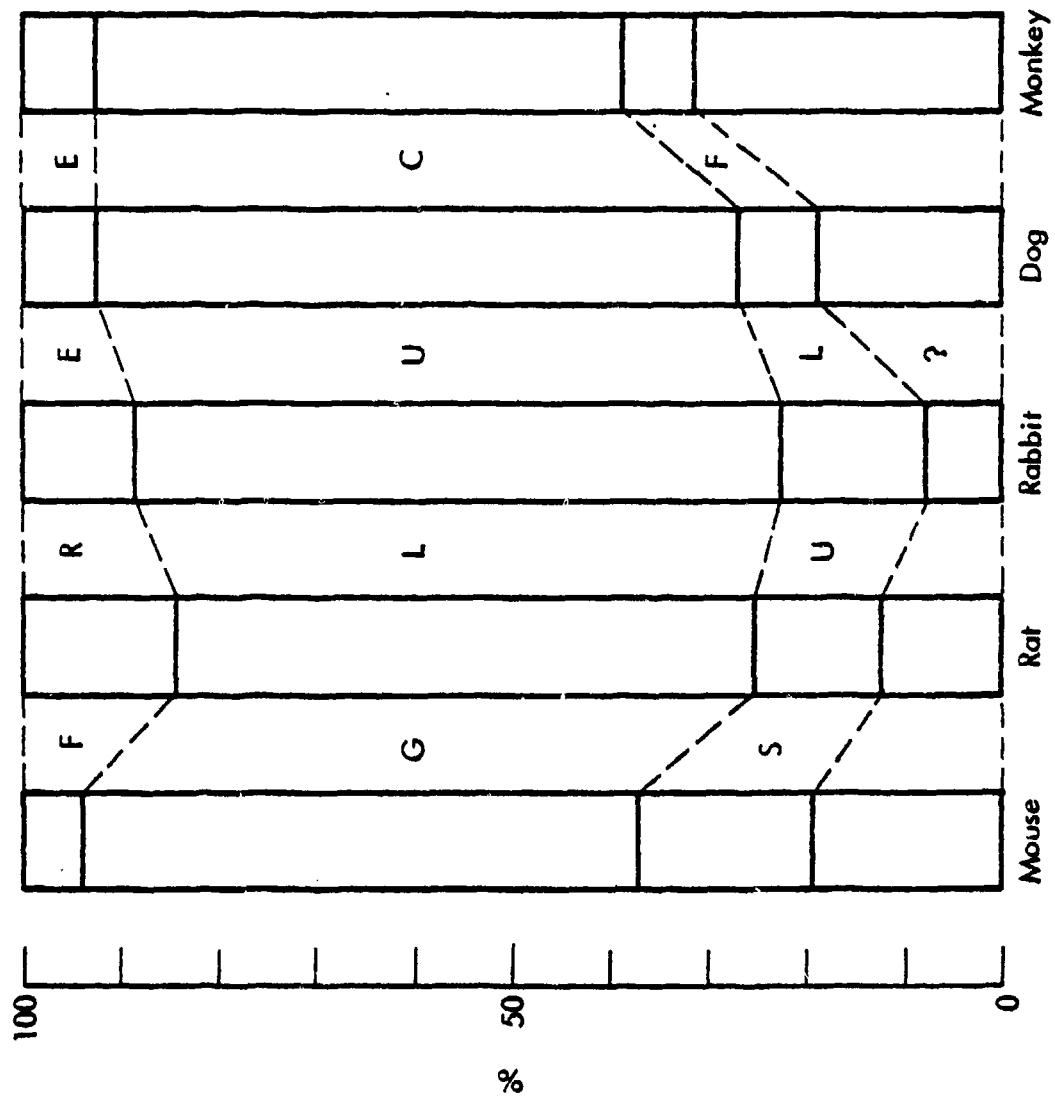


Figure 11 - Urinary Excretory Products of an Oral Dose of 2,4-DNT, shown as Free, Glucuronide Conjugates (gluc), Sulfate Conjugates (sulf) and Unknown Products (?)

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